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Evaluation of bioactivity of QUB1977 from the skin secretion of the frog, Phyllomedusa camba

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Evaluation of bioactivity of QUB1977 from the skin secretion of the frog, *Phyllomedusa camba*

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Contents

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Declaration

I declare that the research reported in this thesis in my own work except where acknowledgement has been made. All work was carried out in Molecular Therapeutics Research, School of Pharmacy, Faculty of Medicine, Health and Life Sciences, Queen's University, Belfast.

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Abstract

There are various bioactive peptides in the skin secretions of amphibians, which play an essential role in amphibian survival in consideration of the complicated natural and artificial factors. It is proven that many of these peptides exhibit antimicrobial, anticancer, antiviral and trypsin inhibitory activities and therefore their potential promising therapeutic value has drawn great attention from numerous researchers

In this study, a novel antimicrobial peptide was isolated and identified from the skin secretion of the frog, *Phyllomedusa camba*. A "shotgun" cloning technique was used to select the biosynthetic precursor sequence and a cDNA library was constructed for amplification of the target sequence. The peptide was isolated and identified using HPLC, MALDI-TOF mass spectrometry.

The synthetic replicate exhibited a broad spectrum antimicrobial activity against Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli* and the yeast *Candida albicans* at concentrations of 4, 32 and 4 μM, respectively. The haemolysis of this peptide was not significant at low concentrations but had a considerable increase at high concentrations. The concentration of the test peptide which induced 50% haemolysis (HC_{50}) was 23 μM. The results indicated that the discovered peptide can be potential drug candidates for the design of new and valuable anti-infective agents.

Chapter 1

Introduction

Introduction

Amphibians which have evolved a wide range of morphological and ecological types are among the most fascinating and numerous of terrestrial vertebrates. They represent the transitional types of vertebrates from fully aquatic fishes to terrestrial amniotes. In the successful attainment of independence from water and colonisation of land, amphibians have undergone a remarkable adaptive radiation. Their skin which is exposed to the constantly-changing environment, helps them defend against the invasion by many microorganisms, parasites and even predators. From this view, the skin of amphibians provides useful information about promising functional molecules and has potential clinical applications.

1.1 Factors affecting the living conditions of amphibians

Since the first International Reptile Congress in 1989, the decline in amphibian populations has become a hot topic for many reptile zoologists. Especially in natural habitats such as national parks, nature reserves and some mountainous areas, the decline is obvious and has aroused widespread concern by reptile zoologists (Barinaga, 1990; Wyman, 1990). By 1993, more than 500 frogs and salamander species had been classified as declining and groups needed to be protected (Alford & Richards, 1999). The data provided by Amphibia Web (Web, 2009) showed that more than 200 amphibian species worldwide have experienced a recent decline in population, including 32 species extinctions, and most of these species have been

extinct in the past two to three decades.

Amphibians are more sensitive to the environment and have higher requirements for external conditions than other land or water animals. Their growth state can be used as a measure of the quality of the global ecosystem and a natural indicator of the biological health of the Earth. In other words, as an important part of the ecosystem, the decline in the number of global amphibian species will have an important impact on other organisms (Blaustein, Wake and Sousa, 1994).

Since there are rich resources in amphibians and we can take advantage of them for medical use, more attention should be paid to their living conditions. Generally, amphibians are vulnerable to a lot of other factors including pathogens (Blaustein et al., 1994), UV-B radiation (Häkkinen et al., 2001), pesticides (Baker et al., 2013), and exotic predators (Adams, 2000; Cruz et al., 2008).

One of the main causes associated with the alarming decline and extinction crisis of amphibians worldwide is chytridiomycosis. The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) bears most of the responsibility for this amphibian disease. Scheele reported that chytridiomycosis resulted in severe age structure truncation, which sequently increased vulnerability to other sources of mortality (Scheele, 2016).

Besides that, habitat loss is also the primary cause of amphibians' numbers falling

(Sodhi et al., 2008). Following the forest clearing, amphibian abundance has suffered dramatic reductions. Meanwhile, although under laboratory conditions, it has been proven that pH becomes a lethal weapon for amphibian embryos of many species (Pierce, 1985), the side effects of acidification are hard to quantify in field populations. Therefore, individual acidification has not been taken as a major cause of widespread amphibian decline since many probable causes can contribute to the decline both at small and large scale (Beebee & Griffiths, 2005). To some degree, environmental perturbations like extensive drought and heavy precipitation and flooding can have effects on species distributions. What is more, biocides like methylisothiazolinone (MIT) impair developmental wound repair and tissue regeneration. This combination of factors leads to a poor condition for the surviving of amphibians.

Considering the factors affecting the living conditions of amphibians and the dramatic decline of amphibian species, carrying out amphibian research is becoming more and more urgent and necessary. It is essential to promote the comprehensive utilisation of amphibians in the food and medicine industries. At the same time, problems like improving active material extraction technologies, obtaining the substances of interest in non-harmful ways and promoting the recycling of resources, remain to be solved.

1.2 Applications of anurans

Anurans, namely frogs and toads, play a distinctive role in *materia medica.* Traditionally, their skin and skin secretions have been used for regulating internal functions or treating dog bites (Costa-Neto, 1998). In traditional Chinese medicine, *venenum bufonis* (Figure 1), skin secretions from the toad ear glands and epidermal glands, has been used for thousands of years and has significant cardiotonic, anti-cancer and anti-radiation effects along with stopping pain and inflammation. What is more, in modern medicine, *venenum bufonis* can be used as an anaesthetic and for the treatment of oesophageal cancer. Documents also show that skin secretions from the giant leaf frog (*Phyllomedusa bicolor*) have positive effects in Alzheimer's disease and other diseases such as depression, stroke and seizures (Amato, 1992).

People in Nagaland, India, have a custom to use frog meat to cover wounds on the skin to treat local ulcers and other diseases (Purna Sai, 1995). In some South American countries like Brazil and Colombia, the shaman healer performs a Kambô ritual on the fresh wounds of the hunters. Skin secretions of *Phyllomedusa bicolor* are applied to the wounds during the ritual which local people firmly believe would bring them strong bodies with luck and strength (Brave, 2014).

Figure 1: Venenum Bufonis

1.3 The diversity of anuran skin peptides

People realised that the amphibian skin contained a large number of amines and active peptides as early as 1976. Indeed, it is hard for other vertebrate tissue to compare with amphibian cutaneous tissue in terms of chemical diversity and concentration of these active substances.

Anuran skin peptides are grouped into neuroactive peptides and cytolytic peptides (König, Bininda-Emonds and Shaw, 2015). These two different peptides are subdivided into distinct families considering their biological functions as well as structural characteristics. Neuroactive peptides are classified as tachykinins, bradykinins, caeruleins, bombesins, opioids, tryptophyllins and miscellaneous peptides. These biologically active components are widely distributed across anurans (Figure 2).

Cytolytic peptides can also be called antimicrobial peptides (AMPs) because of their abilities to inhibit the growth of a wide range of pathogenic microorganisms and even kill them. Meanwhile, some of the cytolytic peptides also show a potent propensity against cancer cells, viruses and as antidiabetic agents (Conlon, 2014). The enormous number of families of AMPs typically share the same genera or they may come from closely related frog species. Amphibian AMPs can be grouped into 7 families including the dermaseptins, the phylloseptins, the plasticins, the dermatoxins, the phylloxins, the hyposins and the orphan peptides (Kastin, 2013).

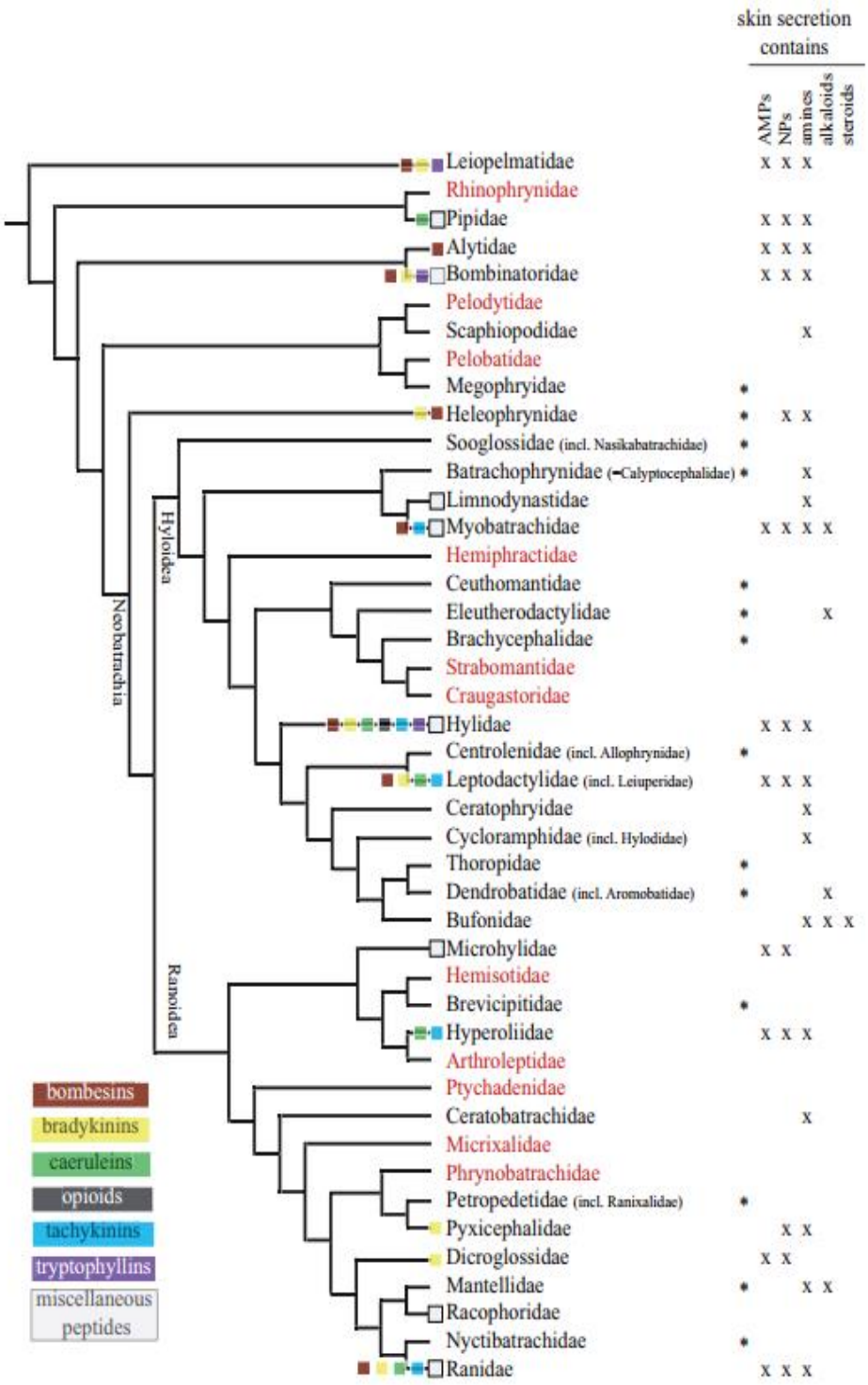

Figure 2: Phylogeny showing the distribution of frog skin defensive chemicals. NPs: neuroactive peptides; x = compound reported from this family; * = not yet investigated specifically for AMPs. Families in red text have been screened, but apparently lack any compounds. (König, Bininda-Emonds & Shaw 2015)

1.4 Amphibian antimicrobial peptides

Amphibians have a wide distribution on continents like South America, Europe, Asia and North America. These numerous species offer a huge variety of bioactive peptides working effectively in the innate immune systems of amphibians. These gene-encoded active peptides can inhibit the productive growth of microbes such as bacteria, fungi and protozoa. What is more, some peptides have been demonstrated to kill cancer cells without showing significant haemolytic activity.

1.4.1 Structures and characteristics

Basically, there are four distinct classifications according to the structures of amphibian AMPs: (a) linear α-helical peptides lacking cysteines (Figure 3); (b) the cysteine-free linear peptides containing a high proportion of residues such as proline or arginine; (c) peptides carrying one disulphide bridge forming a C-terminal loop; (d) Peptides containing several disulphide bonds form structures that are predominantly β-sheet. Disulphide bonds (Figure 4) are very useful for stabilising the tertiary structure of protein molecules.

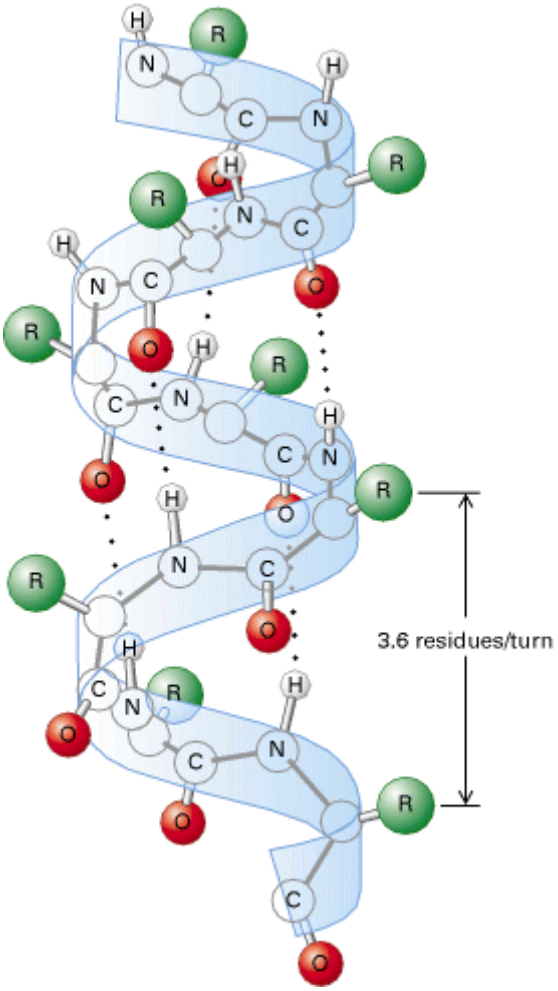

Figure 3: The structure of an α -helix
(from: http://www.nslc.wustl.edu/courses/bio2960
/labs/02Protein_Structure/PS2011.htm)

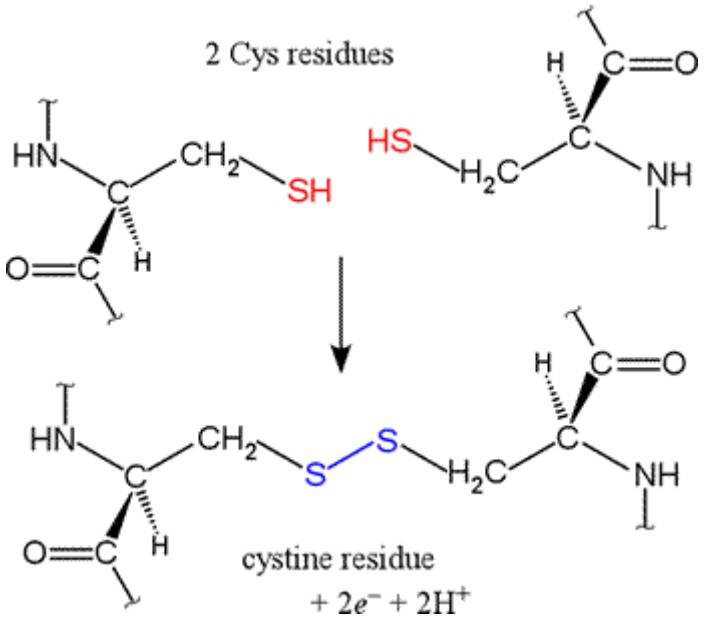

Figure 4: Disulfide bond
(from: https://ptetchem.wordpress.com/notes/disulfide-bond-a2/)

AMPs are normally ribosomally-synthesised and are made up of 10–50 amino acids (Rinaldi, 2002). Most are synthesised in the form of pre-pro-peptides including a signal sequence and a C-terminal cationic peptide (Reddy, 2004). The most obvious feature of an amphipathic α-helical peptide is the hydrophobic and cationically charged surfaces. The mass ranges from 1 kDa to 10 kDa (Rash, 2011). AMPs have different structures like linear, cyclic and open-ended cyclic (Daffre, 2008). The existence of disulphide bridges may result in functional changes. Most AMPs have thermal stability and remain active at 100 ℃for 10-15 min.

1.4.2 The applications of antimicrobial peptides

Antibiotic resistance leads to an increase in human morbidity and medical costs and is therefore considered as a major problem in global public health. Fortunately, some AMPs that are produced in epithelial cells or secreted by skin granular glands may become potential therapeutic agents. These AMPs are potential antibiotics that act quickly and have broad spectra of activities.

Some of the peptides have been shown to be active against Gram-negative and Gram-positive bacteria, fungi, enveloped viruses and even cancer cells. In fact, some peptides have been used for clinical treatment of impetigo, diabetic foot ulcers and *Helicobacter pylori* infections (Reddy, 2004).

Most of the chemical drugs often inhibit both cancer cells and normal cells with greater side effects. However, AMPs can specifically inhibit the growth of certain tumour cells, while being harmless to normal human cells. Cecropins, rat AMPs NP21 and NP22 and the human AMP, HNP21, all show cytotoxic activity against tumour cells such as fibroblasts, cervical cancer cells and lung cancer cells (Sang, Zhang & Zhuge, 2017).

In recent years, AMP applications have gradually become hot spots in biology, medicine, agricultural science, physiology and other areas of research. They also have various applications in agricultural production, the food industry and so on.

- **Animal husbandry**

Due to the extensive use of traditional antibiotics, on the one hand the micro-ecological balance of the animals' gut is seriously damaged; on the other hand, it is easy to stimulate the production of resistant strains, leading to more complex livestock and poultry diseases, seriously affecting the quality of animal products and human health (Khachatourians, 1998). Broad spectrum antimicrobial agents take full advantages in this area.

AMPs can prevent diarrhoea of piglets and improve their adaptation to the fodder and environment (Wang et al., 2004). An AMP used in broiler chickens can promote growth, improve immunity and the efficiency of fodder. Best of all, these peptides have no toxic side effects, no drug residues and even do not pollute the environment. Therefore, they are likely to replace antibiotics and become a new type of food additive.

- **Plant industry**

AMPs also have a broad application prospect in improving antimicrobial abilities of plants. In Li et al.' study, an engineered magainin analogue peptide was expressed in transgenic tobacco, then the plants could resist the pathogen named *Erwinia carotonora subsp. Carotovora*. This test was based on the fact that Myp30 has the ability to slow growth of spore germination of the oomycete, *Peronospora tabacina*, *in vitro* (Li, 2001).

- **Food industry**

AMPs have the potential value as preservatives in the food industry. These peptides have a strong and rapid impact on the inhibition of microbial growth in terms of a variety of Gram-positive and negative bacteria that are food related. They can be easily digested, and have strong activities in acidic conditions. According to this feature, they are suitable for most acidic foods, especially drinks with a good solubility and stability. Therefore, AMPs are promising food preservatives (Wang, 2011).

Nisin has unique anti-infection properties. It has been widely used as a preservative in dairy products, meat products and canned fruits and vegetables across more than 50 countries and regions. In fact, as early as the 1990s, Delves applied Nisin to milk and broth to extend its shelf life (Delves, 1990). Since then, Hudaa Neetoo also used Nisin and other organic acids to successfully control the harmful effect of *Listeria monocytogenes* on smoked salmon and effectively prolonged its shelf life while maintaining its flavour (Neetoo, Ye and Chen, 2008).

Hisako reported that it is possible to control the growth of bacteria and maintain the flavour and colour of food by adding only small doses of AMPs (nmol / g) in the course of meat processing. Meanwhile, the production of nitrites which are harmful to people's health was also reduced (Hisako, 2004).

- **Aquaculture industry**

With the development of the aquaculture industry, the excrement and the residual baits in the aquaculture are more than the degradation capacity of the water environment itself, resulting in serious pollution of water resources. If using antibiotics to treat the pollution, it will not only cause the ecological imbalance of water environment, but also cause drug resistance. Therefore, effective treatment of water resources can be achieved by using AMPs with both stable and effective antibacterial activities to deal with pathogenic microorganisms in aquatic products (Han & Sun, 2009).

- **Other applications**

A number of tests have been conducted to investigate their activity against diseases such as malaria (Krugliak, 2000) and human immunodeficiency virus (Lorin, 2005). Besides, peptides such as magainin and nisin are also considered as potential contraceptives (Zairi, 2005) since they can be cytotoxic to sperm.

Recently，researchers added therapeutic agents from peptides to biocompatible nanocomposites. These systems have multifaceted advantages when it comes to drug delivery because of their submicron size. For instance, Wang combined AMPs with cholesterol to form cationic nanoparticles (Wang, 2010). The unique nanoparticles

could cross the blood–brain barrier more easily and treat patients carrying fatal cryptococcal meningitis and late-stage HIV infection at the same time.

Based on characteristics like low molecular weight, good water-solubility and low antigenicity, AMPs can be developed for clinical applications of anti-bacterial, antifungal, anti-cancer and as natural contraceptives. AMPs that have specific affinities to membranes can be bound to drugs, so that the drugs can act precisely on target sites.

1.4.3 Cytolytic activities

In addition to inhibiting growth of Gram-positive bacteria, *Staphylococcus aureus*, Gram-negative bacteria, *Escherichia coli* and the yeast pathogen, *Candida albicans*, some peptides can also cause human erythrocyte haemolysis. Red cell damage can accelerate cardiovascular and pulmonary diseases.

Membrane lysis is associated with many mechanisms, such as membrane perforation, destruction, dissolution, etc. (Shai, 1999; 2002). For example, a haemolytic peptide (Ladokhin, 1997) can cause pore formation, and the mechanism of action of cecropin P1 is related to membrane destruction (Gazit, 1995).

1.4.4 Problems exist

At present, the development of green, efficient, low toxicity and broad spectrum antimicrobial agents has attracted global interest. New AMPs have been artificially modified based on natural templates to maximise their antimicrobial activity.

AMPs may cause allergic reactions and many natural peptides are haemolytic. In addition, this activity is also a problem that must be solved. Activities of the larger peptides are closely related to their spatial structures, and even their chirality is also an important factor in activity. AMPs obtained by protein synthesis or protein engineering, although consistent with natural peptides in primary structure, are not guaranteed to be consistent in spatial structures, resulting in differences in activity or even no activity at all (Jin & Liu, 2010).

Besides, stability problems *in vivo* affect the application of AMPs in that protein substances used in the body involve many enzyme chemical reactions. Whether AMPs in the body will quickly inactivate or not is also a problem worth thinking about.

1.4.5 Other aspects

Instead of killing frogs, now researchers tend to obtain skin secretions by two methods which are electrically stimulating the dorsal region and gently massaging the dorsal surface until the secretion becomes visible. Of course, the latter technique is

preferred mainly because it reduces the stress of animals and is a humane thing. On the other hand, this method creates sustainable usage of frogs since the number of frogs is limited and keeps declining during recent years. What is worse, many stressors mentioned before may also lead to the alarming decline and extinction crisis of amphibians.

1.5 Aims and objectives of this thesis

There are not many previous reports on skin secretion peptides of *Phyllomedusa camba*, indicating that many problems remained to be resolved. The situation of drug resistant infections is extremely serious, thus making the best of potential novel AMPs is of great importance. Additionally, such peptides abundant in their source appear to have great market competitiveness and are playing an important role in the study of clinical applications.

In this research, the following aims and objectives were set to be achieved:

1) Using the method of shotgun cloning, to construct a cDNA library derived from skin secretions of *Phyllomedusa camba.*
2) Characterise the structures of novel peptides by mass spectrometry (MS) and identifying structures by Circular Dichroism Spectra.
3) Perform bioinformatics analyses on the peptides through the NCBI-BLAST programme, then compare the differences and similarities to further the recognition of their structures.
4) Synthesise the peptide and purify the replicate through corresponding

methods.

5) Perform functional assays such as antimicrobial assays and haemolysis assays to assess the bioactivities of the new peptide.
6) Gain knowledge of the methods of drug discovery and master how to use a variety of related experimental equipment through the whole research process.

Chapter 2

Materials and Methods

Materials and Methods

2.1 Specimen biodata and secretion harvesting

Specimens of adult *Phyllomedusa camba* (Figure 2.1) (n = 3) were commercially purchased from Mr. Juan Chavez, Venom Peru Company (PeruBiotech E.I.R.L, Huánuco, Peru). The dorsal skin surface was stimulated by gentle transdermal electrical stimulation (6V DC; 4 ms pulse-width; 50Hz) through platinum electrodes for two periods of 20s duration or the skin secretion was obtained via mild squeezing and massaging of the glands. The viscous white skin secretion was washed from the skin using deionised water, snap-frozen in liquid nitrogen, lyophilised and stored at -20 ℃ prior to analysis. All the procedures were subject to ethical approval and carried out under appropriate UK animal research personal and project licenses.

Figure 2.1 *Phyllomedusa camba*

(https://en.wikipedia.org/wiki/Phyllomedusa_camba)

2.2 Molecular cloning

Lyophilised skin secretin was dissolved in stabilisation buffer. Magnetic oligo-dT beads were then employed to isolate the polyadenylated mRNA as described by the manufacturer (Dynal Biotech, UK). The isolated mRNA was converted to more stable cDNA using reverse transcriptase. This cDNA was subjected to 3'-RACE procedures to obtain full-length skin peptide precursor nucleic acid sequence data by employing a SMART RACE Kit (Clontech, UK) as described by the manufacturer. Briefly, the 3'-RACE reactions employed a degenerate sense primer (S1; 5′-ACTTTCYGAWTTRYAAGMCCAAABATG-3′ (Y=C + T, W = A + T, R = A + G, M = A + C, B = T + C + G**)** that was designed to a highly-conserved domain of the 5'-untranslated region of previously-characterised skin AMP-encoding cDNAs from *phyllomedusa* frogs.

2.2.1 mRNA isolation

A Dynabeads® mRNA DIRECT™ Kit (Invitrogen, Lithuania) was utilised in mRNA isolation. The poly A tail of poly A mRNA from the sample can hybridise to the bead-bound oligo-dT and can be eluted from the Dynabeads afterwards.

- **Preparation of skin secretion**

Five mg of lyophilised skin secretion were weighed and transferred into an Rnase-free 1.5 ml tube with 1 ml of Lysis/Binding buffer (Dynal Ltd, UK). Then, the 1.5 ml tube was vortexed for 20 min in total and placed on cold ice for several seconds at intervals of three min to obtain the undegraded mRNA. Finally, the lysate solution was centrifuged at 14,000 × g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany) to remove the debris. The supernatants were prepared and stored cold until the beads were ready.

- **Preparation of Dynabeads® Oligo $(dT)_{25}$ beads**

Two hundred and fifty µl of thoroughly resuspended Dynabeads® Oligo $(dT)_{25}$ beads (5 mg/ml) were transferred into a 1.5 ml RNase-free tube. The tube containing the beads was placed on a magnet rack (Dynal MPC-S) for 2 min. Then, the supernatants were removed and discarded as cleanly as possible when the supernatants were clarified. After that, 250 µl Lysis/Binding Buffer were transferred into the 1.5 ml tube to wash the beads by gently shaking. Finally, the supernatants were removed and discarded completely from the 1.5 ml tube on the magnetic rack when the lysate was well prepared to avoid drying the beads and lowering beads capacity.

- **Hybridisation between the poly A tail of mRNA and bead-bound oligo-dT**

The supernatants from the lysate solution were transferred into the 1.5 ml tube containing prepared beads which were combined with oligo-dT on the surface by

covalent binding. The mixture of lysate and beads was blended by slowly and gently shaking for 20 min (2 min shaking and 3 min on ice , repeated four times) at room temperature, which made the poly-A tail of mRNA hybridise to the bead-bound oligo-dT through A-T base pairing. Finally, the tube was placed in the magnet rack for 5 min to make sure that the supernatant was clean and then it was removed. The intact mRNA was isolated from the secretion and retained on the surface of the beads.

- **Washing**

The beads/mRNA complex was washed by slowly and gently using 500 µl Washing Buffer A three times to eliminate impurities. The beads were separated from the washing solution and the washing solution was discarded on the magnetic rack after each washing step. Similarly, the beads/mRNA complex was washed by slowly using 500 µl Washing Buffer B two times to eliminate the lithium salts.

- **Elution**

A volume of 18 µl of cool elution solution (Tris-HCl, 10mM) was added drop by drop into the 1.5 ml tube containing the well-washed beads and the 1.5 ml tube was flicked gently to make every droplet carry the beads down to the bottom until all the solution ran through. Then, the 1.5 ml tube was incubated in a heating block (Grant, Cambridge, UK) at 80℃ for 2 min to remove the mRNA from the beads. All supernatants were subsequently transferred into a 0.2 ml RNase-free PCR tube on the magnetic rack as soon as possible to avoid mRNA readsorbing onto the beads. Finally,

the 0.2 ml PCR tube was cooled on ice for 2 min and then the solution was allocated into five chilled-prepared 0.2 ml PCR tubes which included 4 µl volume for three PCR tubes and 3 µl volume for two PCR tubes, respectively (Figure 2.2.1.1).

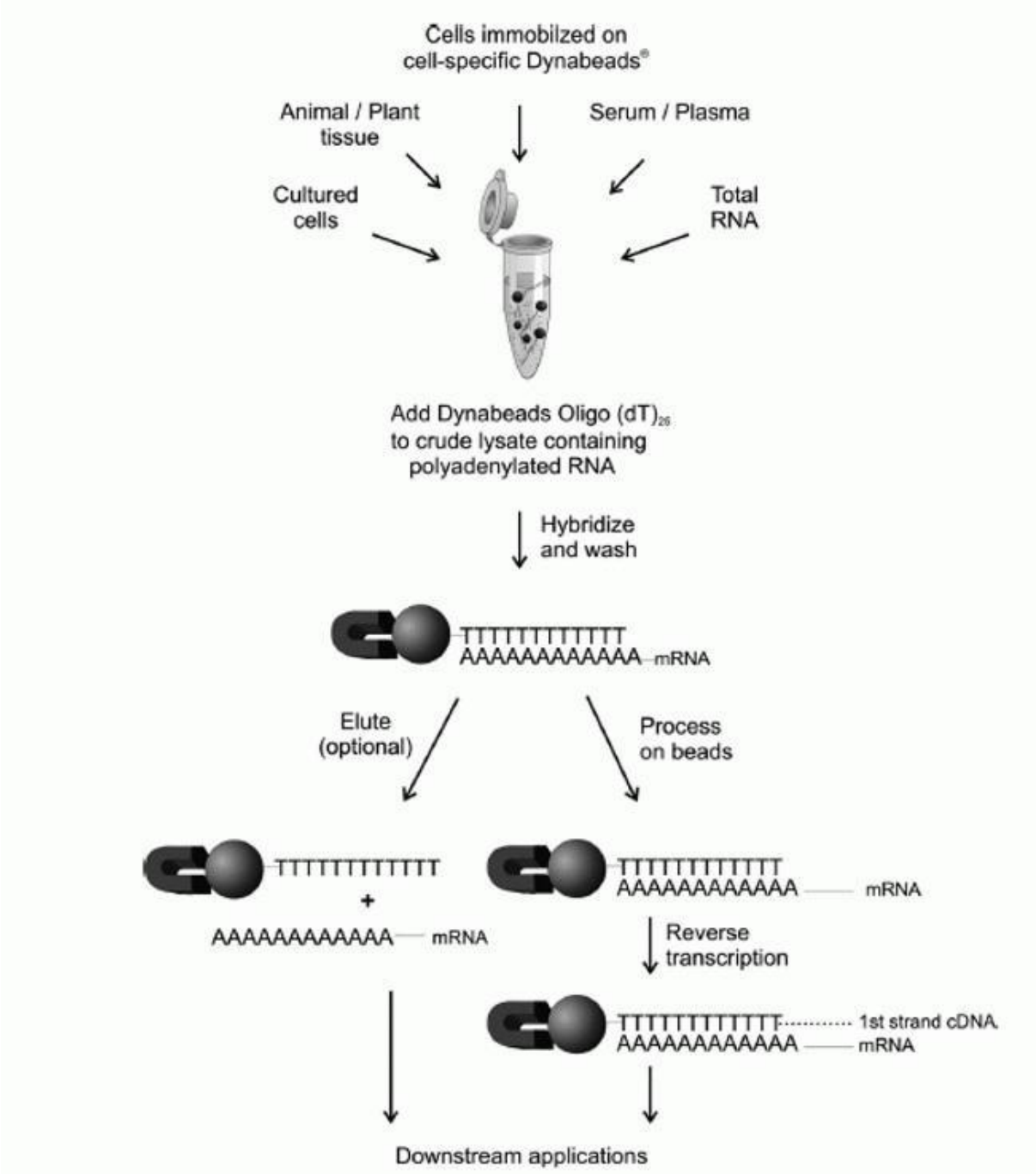

Figure 2.2.1.1 Dynabeads® mRNA DIRECT™ workflow

(https://www.thermofisher.com/order/catalog/product/61011)

2.2.2 cDNA library construction

A BD SMART™ RACE cDNA Amplification Kit (BD Bioscience Clontech, UK) was used in cDNA library construction and primary cDNA amplification. 5' RACE Ready cDNA was synthesised using a 5'-RACE CDS Primer and the BD SMART II™ A Oligonucleotide which contained a terminal stretch of G residues to pair with the dC-rich cDNA tail at the end. 3'-RACE-Ready cDNA was synthesised using 3'-RACE CDs Primer by a reverse transcription reaction.

- **Preparation of sample mixture**

➢ **Preparation of mixture for 3' RACE Ready cDNA synthesis**

The following components were combined and mixed completely by pipetting in three 0.2 ml PCR tubes respectively. One extra volume of reagents was calculated and added to ensure sufficient volume for the RT-PCR reaction (Table 2.2.2.1).

Table 2.2.2.1 The components of the 3' RACE cDNA reaction

Component	Final Volume	Final Concentration
RNA sample	4 µl	40%
3'-RACE CDS Primer	1 µl	1 µM
dNTP Mix (10 mM)	1 µl	1 mM
DTT (20 mM)	1 µl	2 mM
5X First-Strand Buffer,30mM $MgCl_2$	2 µl	1 X
BD Reverse Transcriptase	1 µl	10 Unit/µl

*The Master Mix includes dNTP Mix, DTT and 5X First-Strand Buffer for 5

reactions.

- **Preparation of mixture for 5' RACE Ready cDNA synthesis**

The following components were combined and mixed completely by pipetting in two 0.2 ml PCR tubes. One extra volume of reagents was calculated and added to ensure sufficient volume for the RT-PCR reaction (Table 2.2.2.2).

Table 2.2.2.2 The components of the 5' RACE cDNA reaction

Component	Final Volume	Final Concentration
RNA sample	4 µl	40%
5'-RACE CDS Primer (10 µM)	1 µl	1 µM
SMART II (10 µM)	1 µl	1 µM
dNTP Mix (10 mM)	1 µl	1 mM
DTT (20 mM)	1 µl	2 mM
5X First-Strand Buffer,30mM $MgCl_2$	2 µl	1 X
Reverse Transcriptase	1 µl	10 Unit/µl

*The Master Mix includes dNTP Mix, DTT and 5X First-Strand Buffer for 5 reactions.

- **Reverse transcription polymerase chain reaction (RT-PCR)**

Five PCR tubes, containing sample mixture, were micro-centrifuged and incubated in the heating block at 70℃ for 2 min to combine the primer and templates. Then, five 0.2 ml PCR tubes were cooled ice for 2 min. After that, 4 µl prepared Master Mix was

divided into each 0.2 ml PCR tube and pipetted completely. Subsequently, 1 µl Reverse Transcriptase was added into each 0.2 ml PCR tube and pipetted completely. After adding all solutions, five 0.2 ml PCR tubes were micro-centrifuged to collect all contents at the bottom without bubbles and incubated in the thermal cycler (Applied Biosystems, UK) at 42 ℃ for 1.5 h to complete the reverse transcription reaction.

- **Concentration, dilution and fault correcting**

Fifty µl of deionised water were added into each PCR tube to lower the concentration. Then, five PCR tubes were incubated in the thermal cycler at 72 ℃ for 7 min to correct the faults in the reaction and kill some enzymes such as Reverse Transcriptase. At this point, 3'- and 5'-RACE Ready cDNA templates were obtained and stored at -20 ℃ in the freezer.

2.2.3 Polymerase chain reaction (PCR) amplification of cDNA

- **Preparation of mixture for RACE-PCR reaction**

The following components were combined and mixed completely by pipetting and an extra volume was calculated and added to ensure sufficient volume for the RACE-PCR reaction (Table 2.2.3.1).

Table 2.2.3.1 The components of one RACE-PCR reaction

Component	Volume	Final Concentration

PCR-Grade water	2.6 µl	-
10X BD Advantage 2 PCR Buffer	1 µl	1 X
dNTP Mix (10 mM)	0.2 µl	0.2 mM
NUP Primer (20 µM)	0.5 µl	1 µM
Sense Primer (20 µM) Anti-sense primer	0.5 µl	1 µM
50 X BD Advantage 2 Polymerase Mix	0.2 µl	1 X
3' RACE-Ready cDNA templates	10 µl	

*The 3' RACE-Ready cDNA templates were substituted with water in the negative control.

*The Master Mix includes PCR-Grade water, 10X BD Advantage 2 PCR Buffer, dNTP Mix, NUP Primer, Sense Primer and BD Advantage 2 Polymerase Mix for 4 reactions.

- **3' RACE-Ready cDNA amplification**

Twenty µl of Master Mix were allocated into four well-prepared PCR tubes and 10 µl of 3' RACE-Ready cDNA templates were added into two PCR tubes. 10 µl of PCR-Grade water were added into another two PCR tubes as negative controls. 0.5 µl NUP primer (supplied with the kit) and 0.5 µl specific degenerate sense primer (S1; 5′- ACTTTCYGAWTTRYAAGMCCAAABATG-3′ (Y=C + T, W = A + T, R = A + G, M = A + C, B = T + C + G**)** were added into each PCR tube. The degenerate primer was designed based on the highly-conserved 5'-untranslated region of previously-characterised homologous peptide cDNAs from *Phyllomedusa camba***.** All

of the reagents were pipetted completely and micro-centrifuged to collect all contents at the bottom without bubbles. Finally, the RACE-PCR programme with a gradient temperature was set and commenced in the thermal cycler. The appropriate annealing temperature was determined in the RACE-PCR reaction. Three steps of PCR reaction with different conditions were set up and each cycle included 94 ℃ denaturation for 30 s to obtain single-stranded DNA templates, 58 ℃ annealing for 30 s between primer and single-strand DNA templates and 72 ℃ extension for 3 min. All the procedures were repeated over 40 thermal cycles to obtain double-stranded DNA amplification. The annealing temperature in one group included one 3' RACE-Ready cDNA template and one negative control at 53 ℃, whereas it was set at 55 ℃ in another group. All of the four samples after RACE-PCR reaction were stored at -20 ℃ in the freezer (Figure 2.2.3.1).

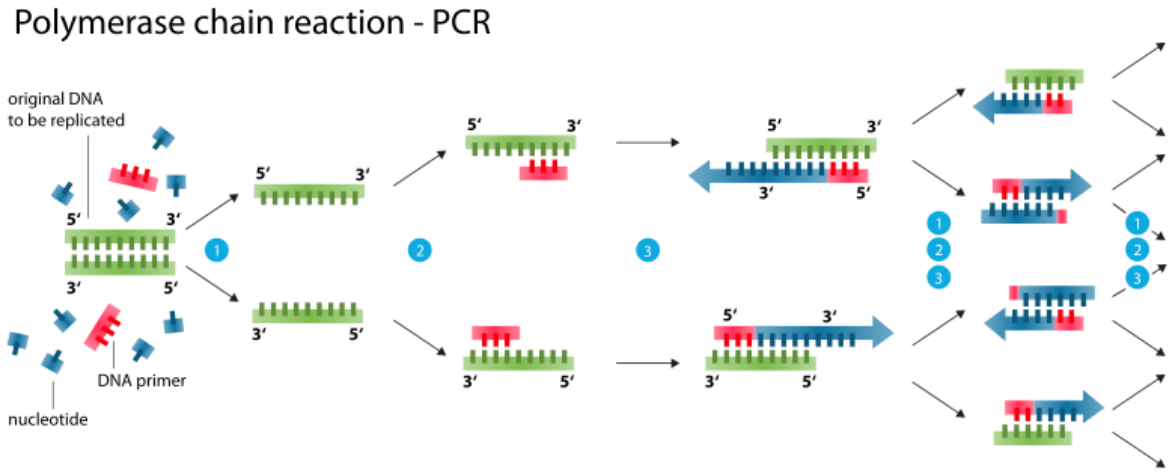

Figure 2.2.3.1 Polymerase chain reaction (PCR)

(https://en.wikipedia.org/wiki/Polymerase_chain_reaction)

2.2.4 Agarose gel electrophoresis

Gel electrophoresis is a technique to separate DNA fragments, based on their sizes and charges.

- **Preparation of 1.5% agarose gel**

0.45 g agarose (Invitrogen, UK) was transferred into a 200ml flask with 35 ml of freshly prepared 1X Tris/Borate/EDTA (TBE) buffer (Invitrogen, UK). The flask was heated in a microwave oven without foaming until all the agarose was dissolved completely, and then it was cooled for several minutes. 2.5 µl of 10 mg/ml Ethidium Bromide (EB) (Sigma-Aldrich, USA) were added into the flask when the agarose had cooled to about 60 °C to bind to the DNA and make the DNA visible under ultraviolet light. The flask was swirled gently to ensure good mixing of contents. Then, the molten agarose was poured into a gel tray which was placed after cooling, into a gel electrophoresis tank with a sample comb. The agarose was then allowed to solidify fully at room temperature for about 40 min. After the gel became solid, the comb was gently removed, then the TBE buffer was poured into the gel tank until just above the surface of the gel.

- **Sample loading and electrophoresis**

Two and half µl of a standard DNA ladder (Invitrogen, UK) composed of several fragments of known molecular weight, were loaded carefully into the first lane of the agarose gel to measure the size of DNA fragments. 1.5 µl of samples and 0.5 µl of

loading dye (0.25% bromophenol blue, 15% Ficoll 400 in TAE) were mixed well and loaded carefully in the other lanes in order. After that, the electrophoresis was run at 90 v and the samples travelled through the gel from the negative electrode to the positive electrode for 30 min until the yellow colour indicator reached two-thirds of the gel. Finally, the power was stopped and the gel was transferred into a large weigh boat for later detection of bands.

- **Detection of bands and gel analysis**

The electrophoresis gel was placed under the UV trans-illuminator BioDoc-It® Imaging System (NVP, Cambridge, UK) and a photographic image was recorded as the result. The DNA bands of the samples were compared with those of the ladder to determine whether the DNA amplification was successful or not. The 1X TBE Buffer was recycled and the samples were stored at -20 ℃ in the freezer.

2.2.5 PCR product purification

An E.Z.N.A.® Tissue DNA Kit (Omega, Norcross, UK) was employed in PCR product purification, in which DNA was bound to silica-based filter membranes during washing steps and eluted for collection (Figure 2.2.5.1).

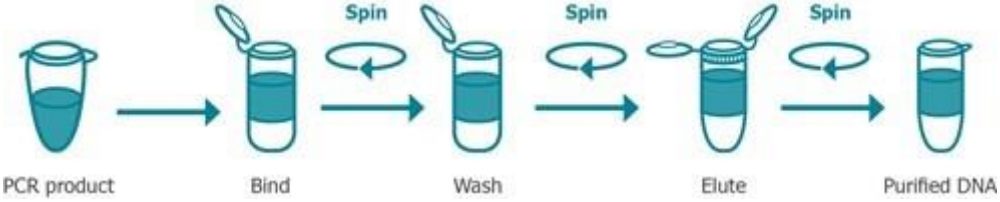

Figure 2.2.5.1 The workflow for PCR product purification

(https://www.sourcebioscience.com/products/life-science-research/molecular-biology-tools/nucleic-acid-purification/resource-pcr-purification-kit/)

- **DNA binding with the filter membranes**

The DNA amplification samples from the previous steps were mixed together and transferred into a 1.5 ml DNase-free tube. Then, 87 μl Buffer CP were added into the 1.5 ml tube where five volumes Buffer CP corresponded with one volume PCR product according to the weight of DNA product. After pipetting evenly and completely, the liquid in the PCR tube was transferred into the center of the cartridge. The cartridge was centrifuged at 8000 × g for 1 min. The liquid was discarded and 700 μl Washing Buffer was added into the cartridge. Then the cartridge was centrifuged at 8000 × g for 1 min. The liquid was removed and 500 μl Washing Buffer was added into the cartridge. Then the cartridge was centrifuged at 8000 × g for 1 min.The liquid was discarded and the cartridge was centrifuged at 8000 × g for 2 min to remove the ethanol completely. 30 μl of distilled Water was added to the center of the cartridge and a sterile 1.5 ml tube was used to hold the contents. The tube was incubated at room temperature for 2 min and then centrifuged at 15000 × g for 1 min. The tube contents were concentrated in the concentrator for 50 min. Then the sample was stored in the -20 ℃ freezer covered with parafilm prior to further analysis.

2.2.6 Ligation

A pGEM®-T and pGEM®-T Easy Vector (Promega, USA) kit was used for ligation, transformation, blue and white colony screening and isolation of recombinant DNA reactions. The DNA with A at both ends of the strand could bind to and insert into the site of the pGEM®-T Easy Vector (50 ng/μl) with T through A-T based pairing.

1) 7 μl of PCR reagents were added into the tube containing the lyophilized DNA with gentle pipetting.
2) 2.5 μl 2X Rapid Ligation Buffer, 0.5 μl T4 DNA Ligase and 0.5 μl pGEM®-T Easy Vector was added into a sterile PCR tube. The pGEM®-T Easy Vector was briefly centrifuged before use.
3) 1.5 μl DNA sample from 1) was added into the mixture from 2) with gentle pipetting.
4) The tube was then incubated at room temperature for 1h and then stored at 4℃ overnight.

2.2.7 Transformation

The recombinant vectors were transformed into the competent cells and selected by ampicillin, IPTG and X-Gal using the pGEM®-T and pGEM®-T Easy Vector kit (Promega, USA).

- **Preparation of LB/ampicillin/IPTG/X-Gal plates**

LB agar (Invitrogen, UK) was weighed and dissolved in 200 ml double deionised water in a 400 ml glass bottle. Then, the bottle was autoclaved for sterilisation. 550 µl ampicillin (Roche, USA) were added into the heated agar solution and mixed completely. This was to select the bacteria with the specific antibiotic resistance gene. Then, 11 ml of melted agar solution was aliquoted and poured into each Petri dish. After the solidification of agar, 100 µl IPTG (Promega, USA) as an inducer were added and spread over the surface evenly which induced the expression of the lacZ gene. Subsequently, 20 µl X-Gal (Promega, USA) as a chromogenic substrate, were added and spread over the surface completely in a dark environment and this reacted with β-galactosidase and was detected by a colour change. Finally, all the plates were incubated upside down keeping the water inside the agar for 45 min at room temperature, and then the plates were ready for cell or bacterial culture. In addition, the LB agar plates could be stored at 4 ℃ for up to one month.

- **Transformation**

1) Ligation product were transferred into a sterile 1.5 ml tube and then set on ice.
2) JM109 competent E. coli cells were removed from the -80 ℃ freezer and immediately placed on ice for 4 min to thaw.
3) 50 µl of JM109 cells were added into a 1.5 ml tube containing ligation product with no pipetting. Then the tube was incubated on ice for 20 min.
4) The tube was then placed in a heating block for exactly 47 s and then set on ice for

2 min.

5) 950 µl of Super Optimal Broth (SOC) Medium were added to the mixture gently. Then the tube was incubated at 37 ℃ for 150 min.

6) A solution of 6.4 g LB Agar in 200 ml DD water was made and then autoclaved.

7) 12 ml of agar solution were added into Petri dishes then these were incubated at 37 ℃ for 30 min.

8) 100 µl isopropyl-β-d-thiogalactoside (IPTG) and 20 µl Xgal were added onto each plate and a "hockey stick" was used to spread the reagents.

9) 100 µl of SOC solution were added onto each dish and spread evenly prior to incubation at 37 ℃ for 24 h.

2.2.8 Blue and white colony screening

There were three kinds of colonies found growing on the LB/ampicillin/IPTG/X-Gal plates from the previous procedures including white colonies, blue colonies and white colonies with a blue dot. Pure white colonies and white colonies with a blue dot, in which the vectors were inside the competent cell and contained the recombinant DNA, were selected and subcultured in the solid medium for further identification. The 1.5 cm length squares were divided by drawing lines at the bottom of the Petri dish, then the pure white colonies and white colonies with blue dots were picked up and transferred into three new LB/ampicillin/IPTG/X-Gal plates by streaking without touching the edge of the lines using an inoculating loop under a sterile environment.

All of the three plates were incubated upside down at 37℃ overnight (16-24 h) for subculture and further selection.

2.2.9 Isolation of recombinant DNA by cloning PCR

A single DNA was isolated and amplified by the cloning PCR reaction, in which the M13 Forward Primer (5'-GTAACGCCAGGGTTTTCCCAG-3') and M13 Reverse Primer (5'-TGTGAGCGGATAACAATTTCAC-3') bound to the 5' and 3' ends of inserted DNA, respectively by use of an Advantage® 2 PCR Kit (Clontech Inc, USA).

1) 20 µl of PCR water were added into autoclaved 0.5 ml tubes and a sample from each white colony was transferred into these tubes.
2) The tubes were incubated at 100℃ for 5 min and then set on ice for another 5 min.
3) Then the mixtures were centrifuged at 8000 × g for 5 min.
4) A master mix was then made (Table 2.2.9.1).
5) The master mix was placed in PCR tubes. Then 2.5 µl of supernatant from each sample were added into each tube.
6) 0.25 µl of Taq Polymerase was added into each PCR tube. The contents of tubes were gently mixed by pipetting.
7) The tubes were then incubated in a PCR machine (Applied Biosystems, USA) for 32 cycles of 94 ℃ initial denaturation for 30 s, 55 ℃ annealing for 30 s and 72 ℃ extension for 3 min.

Table 2.2.9.1 Master mix for PCR of Plasmid cDNA

Component	Final Volume	Final Concentration
dNTP Mix	1 µl	0.2mM
PCR-Grade water	31 µl	-
Cloning PCR Buffer	10 µl	1 X
M13 Forward Primer(20µM)	2.5 µl	1 µM
M13 Reverse Primer(20µM)	2. 5 µl	1 µM

2.2.10 Agarose gel electrophoresis analysis

1) 0.45g agarose was mixed with 35 ml 1XTBE Buffer. The mixture was rotated and heated in a microwave until transparent.
2) 2.5 µl EB was then added into the agarose.
3) The agarose was then poured into the grooves of the gel analysis device. All the bubbles in the mixture were carefully removed.
4) 40 min later, 2.5 µl of DNA ladder were added into the first well. 1.5 µl from each sample tube were added into separate wells.
5) The gel analysis device was run at 90 V for 30 min, after which, the power was turned off and the gel was viewed under a UV light. Samples were then selected for purification.

2.2.11 Selected PCR product purification

An E.Z.N.A. ® Tissue DNA Kit (Omega, Norcross, UK) was employed in PCR product purification, in which DNA was bound to silica-based filter membranes during washing steps and eluted for collection.

1) Selected DNA products were transferred into sterile 1.5 ml tubes and mixed with 250 µl CP buffer.
2) The liquid was transferred into the center of the cartridge. Then the cartridge was centrifuged at 8000 × g for 1 min.
3) The liquid was discarded and 700 µl Washing Buffer was added into the cartridge. Then the cartridge was centrifuged at 8000 × g for 1 min.
4) The liquid was removed and 500 µl Washing Buffer was added into the cartridge. Then the cartridge was centrifuged at 8000 × g for 1 min.
5) The liquid was removed and the cartridge was centrifuged at 8000 × g for 2 min to remove the ethanol completely.
6) 20 µl of DD Water were added to the center of the cartridge and a sterile 1.5 ml was used to hold the contents. The tube was incubated at room temperature for 2 min and then centrifuged at 8000 × g for 1 min.
7) The tube contents were then concentrated in a concentrator for 40 min. Then the sample was stored in the -20 ℃ freezer covered with parafilm.

2.2.12 DNA sequencing reaction

A BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) was utilised in the DNA sequencing reaction in which the sequence was detected by an ABI 3730 automated sequencer (Applied Biosystems, USA).

- **Preparation of mixture for sequencing PCR reaction**

Four optimal DNA samples were chosen for the sequencing reaction and 10 µl of M13 Forward Primer were diluted in 52.5 µl PCR-Grade water. The following components were combined and mixed completely by pipetting, and an extra volume was calculated and added to ensure sufficient volume for the sequencing PCR reaction (Table 2.2.12.1).

Table 2.2.12.1 The components of a sequencing PCR reaction

Component	Final Volume	Final Concentration
PCR-Grade water	12.4 µl	-
5X Sequencing Buffer	3.57 µl	1 X
M13 Forward Primer	1.14 µl	0.8 µM
Terminator Ready Reaction Mix	2.86 µl	13.68%
DNA template	2.5 µl	10-1000 ng

*The Master Mix includes PCR-Grade water, 5X Sequencing Buffer, M13 Forward Primer and Terminator Ready Reaction Mix for 4 reactions.

- **DNA sequencing reaction**

Master Mix and 2.5 µl sample were aliquoted into 0.2 ml PCR tubes and the sequencing PCR reaction was set and commenced using the following programme. Each cycle in the thermal cycler consisted of: 96℃ denaturation for 20 s, 55℃ annealing for 10 s and 60℃ extension for 4 min. Cycles were repeated 26 times in total for 2h 15min. Finally, the four sequencing products were stored at -20℃ in the freezer.

2.2.13 Extension product purification by ethanol

- **Reagent preparation**

One ml PCR-Grade water and 19 ml ethanol were mixed completely to produce a 95% ethanol preparation and 6 ml PCR-Grade water and 14ml ethanol were mixed for the 70% ethanol preparation.

- **Ethanol purification**

Seventy-two µl of 95% ethanol were added into the PCR tube with sequencing reaction products and pipetted vigorously. After that, all the solutions were transferred into a 1.5 ml tube with 10 µl PCR-Grade water. Each of the 4 tubes was vortexed for 30 s and kept at room temperature for 20 min and then centrifuged at the maximum speed of 8000 × g for 20 min. Immediately after this, the supernatants were discarded as cleanly as possible. Similarly, 260 µl 70% ethanol were added into each 1.5 ml tube with sequencing reaction products and mixed, vortexed for 30 s and centrifuged

again as before. Then the supernatants were discarded quickly. Afterwards, a 1 min cooling step and a 1 min heating step at 95℃ were repeated 3 times. Finally, the contents of the 1.5ml tubes were concentrated for 3 h to dry the DNA and to drive the ethanol away. Finally, the 4 samples were stored at -20℃ in the freezer.

2.2.14 Sequencing

Ten μl HiDi (highly deionised-formamide) were added to each DNA sample which had been concentrated for 1 h before use. Then, the 1.5ml tubes were vortexed and then centrifuged briefly as before. Afterwards, the tubes were heated to 95℃ for 4 min in the heating block and cooled on ice for 3 min. Subsequently, 9 μl of well-prepared mixture sample was loaded into the 96-well plate in odd or even rows. Finally, the sequencing results were obtained using an ABI 3730 automated sequencer (Applied Biosystems, USA). The elongation of DNA strands in the solution was terminated by the modified ddNTPs randomly and detected by fluorescence.

- **Sequence analysis**

The exact recognition site with the sequence AATTCGATT from the pGEM®-T Easy Vectors and a series of guanosine endings were recognised in the results. The middle sequences were regarded as targeted sequence and translated into amino acid sequence on the website Expasy (http://web.expasy.org/translate/). At this point, the putative nucleotide and translated open reading frame amino acid sequences of cloned

cDNA encoding precursor were obtained.

2.3 Solid phase peptide synthesis

The novel mature peptide was chemically-synthesised by solid phase Fluorenylmethoxycarbonyl (Fmoc) chemistry in a Tribute automated solid-phase synthesiser (Protein Technologies, Inc, Tucson, AZ, USA). The mechanism of SPPS is one of repeated cycles of deprotection-wash-coupling-wash (Figure 2.3.1). The unequivocal primary structure of the novel peptide was as follows: FLSLIPKIATGIAALAKHL-NH_2.

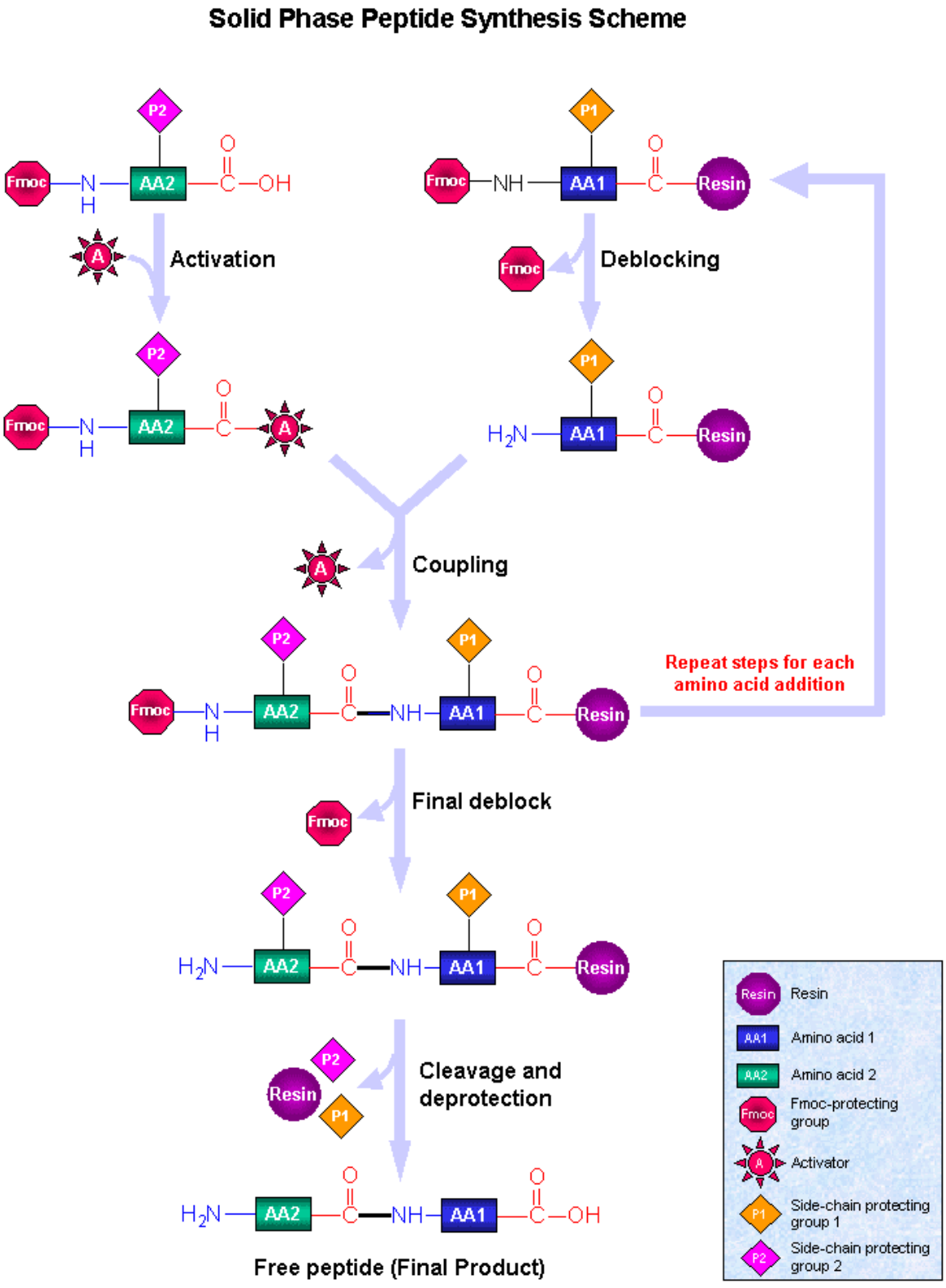

Figure 2.3.1 The scheme of solid-phase peptide synthesis

(http://www.sigmaaldrich.com/content/dam/sigma-aldrich/life-science/custom-peptides/solid-phase-synthesis.gif)

2.3.1 Peptide synthesis

A Tribute Peptide Synthesiser (Protein technologies, USA) was used for solid-phase peptide synthesis (Figure 2.3.1.1). Before the carousel was loaded with amino acid vials, the inline solvent filters and source of nitrogen needed to be checked as were the reagent bottles to ensure that sufficient reagents were present for the synthesis. The bottles needed to be pressurised before the reagent vessels and amino acids were loaded. Synthesis was started through the pressing of the run button after the coupling programme was initiated.

Figure 2.3.1.1 The Tribute peptide synthesiser

(https://static1.squarespace.com/static/55a71e84e4b03f10bdc2d0e5/t/55c7aeb2e4b0ff8d2113dfc7/1439149756438/)

The weights of catalyst 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and dry amino acids in equal proportion and in four-fold molar excess, were calculated and weighed into acetone-cleaned amino acid vials. Then, 0.3 mmol rink amide resin which contained an amide group for the C-terminus was also weighed into the reaction vessel. During the peptide synthesis reaction, the reaction vessel and pipeline were washed by dimethylformamide (DMF) first, and then the Fmoc protecting groups were deprotected using 20% (v/v) piperidine in DMF. Each amino acid residue was activated and coupled using 11% (v/v) N-Methylmorpholine (NMM) in 89% (v/v) DMF combined with activator HBTU. After that, the peptide was synthesised from C-terminal to N-terminal by the PS4 synthesiser. Finally, degassed dichloromethane (DCM) was employed for washing the peptide/resin complex after the synthesis reaction. The peptide/resin was dried in a vacuum desiccator overnight.

2.3.2 Peptide cleavage and rotary evaporation

1) The dried resin was weighed and transferred into a 50 ml round-bottomed flask.
2) 28.2ml 94% (v/v) trifluoroacetic acid (TFA), 0.6ml 2% (v/v) ethanedithiol (EDT), 0.6 ml 2% (v/v) water and 0.6 ml 2% (v/v) thioanisole (TIS) were added into the 50 ml round-bottomed flask to deprotect the protecting groups of side chains including Trp (W).

3) The flask was removed to a magnetic stirrer for 2.5 h at room temperature.

4) After stirring, the cleavage solution was filtered using a Buchner funnel into a 50 ml round-bottomed flask and then the resin was washed by a volume of DCM.

5) The filter liquid was concentrated by a rotary evaporator in a water bath until 4-5 ml remained.

6) The concentrated liquid was transferred to a 50 ml centrifuge tube.

7) 45-50 ml of Et2O was added to the tube and the tube was left covered by silver paper with holes at room temperature, to complete the oxidation reaction for 3 days to form the disulphide bonds if required (the tube was shaken 2-3 times/day and 50 ml of Et2O were added).

2.3.3 Peptide washing

1) The tube was centrifuged at 5000 × g for 5 min and the supernatant was discarded.

2) A volume of Et2O was added to the tube and then the tube was centrifuged. This was repeated three times.

3) The supernatant was discarded and the tube was covered by silver paper with holes and placed in a vacuum desiccator to dry overnight.

2.3.4 Peptide lyophilisation

1) 30 ml of Buffer B (0.05%/29.95/70.0 (TFA/water/Acetonitrile) (v/v/v)) were added to the tube which was then vortexed to dissolve the peptides.

2) The tube was then frozen in liquid nitrogen for 10-20 min.

3) This tube was then placed into an Alpha 1-2 freeze-drying system (Martinchrist, Germany) to be lyophilised for 72 h.

4) The lyophilised peptide was stored at – 20 C.

The molecular weight of the peptide was calculated by Peptide Property Calculator (https://www.genscript.com/tools/peptide-property-calculator).

2.4 Reversed-phase high performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is widely used to separate, identify and quantify each compound in a mixture through use of a stationary and a mobile phase. HPLC works through pumping a pressurised liquid mobile phase and a sample mixture through a column filled with a solid phase stationary phase. Depending on different absorptive characteristics, the components of the sample mixture are separated from each through interaction with the solid support and elution at different concentrations of mobile phase.

Reverse phase HPLC (RP-HPLC) was employed to separate the skin secretion components into fractions. The RP-HPLC contains a non-polar stationary phase and an aqueous, moderately polar mobile phase. The mechanism of RP-HPLC is hydrophobic interaction between sample components and stationary phase. A representative HPLC system consists of pumps, a sampler, column, detector, fraction

collector and data analysis system (Figure 2.4.1).

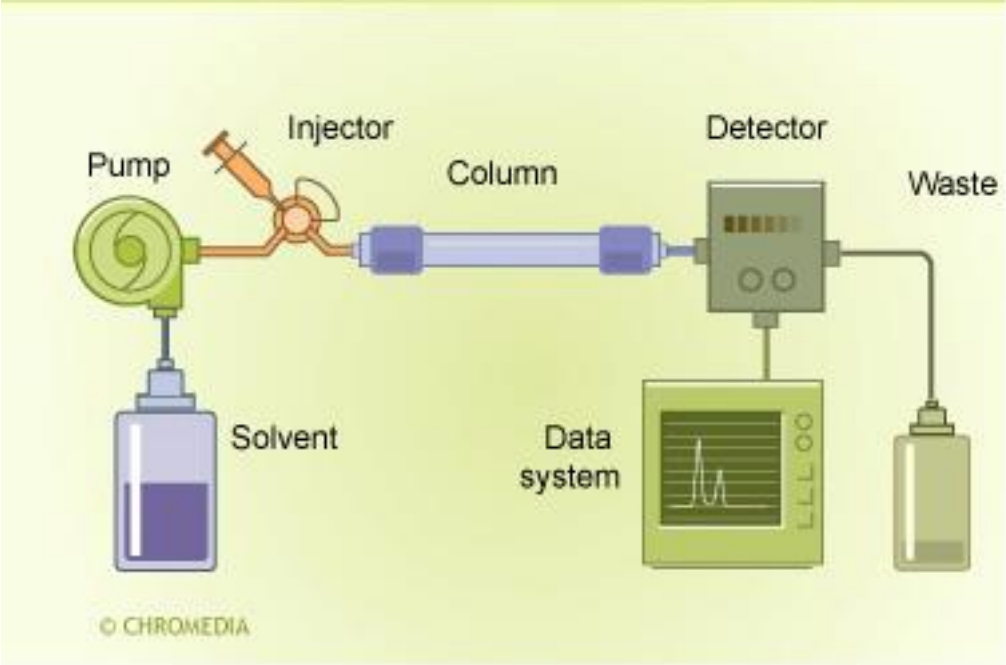

Figure 2.4.1 A typical HPLC system

(http://www.chromedia.org/chromedia?waxtrapp=yoovpDsHonOvmOlIEcCI&subNav=yarwnEsHonOvmOlIEcCIbB)

10 mg of crude lyophilised peptide was weighed and diluted in a 15ml universal tube with 5 ml Buffer A and 5 ml Buffer B. Then, the 15ml universal tube was vortexed and centrifuged at the maximum speed for 15 min. The clear supernatants were transferred into another 15ml universal tube. An analytical reverse phase HPLC Jupiter C5 column (250nm * 4.6 mm, Phenomenex, UK) was washed with Buffer B for 30 min and equilibrated in Buffer A for 30 min before use. Subsequently, 1 ml of clear supernatant was pumped onto the Jupiter C5 column on a Cecil Adept CE4200 HPLC system (Cecil, Cambridge, UK) for peptide elution and purification with 214nm wavelength detection. The peptide was eluted from the column with a linear

gradient from 100% Buffer A to 100% Buffer B over 80 min at a flow rate of 1 ml/min. The fractions were collected in polypropylene tubes (Sarstedt, Germany) at every peak using an Amersham Biosciences Frac-920 fraction collector and utilised for identification.

2.5 Peptide analysis by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry

MALDI-TOF mass spectrometry was used for peptide identification by mass analysis using a linear time-of-flight mass spectrometer (Voyager DE, PerSeptive Biosystems, Framingham, MA, USA) in positive detection mode. Internal mass calibration was verified using the standard peptides corresponding with standard molecular masses to ensure high accuracy of ±0.1%. 2 µl of HPLC fractions were loaded and spotted onto the MALDI ground-steel target plate, and 1 µl excess matrix solution (10 mg/ml) which contained alpha-cyano-4-hydroxycinnamic acid (CHCA) diluted in acetonitrile/TFA/water (70/0.02/30, v/v) was also loaded and spotted after drying the fractions. The samples were ionised and flew through the electrical field in the instrument to reach the detector. The peptide was identified by mass measurement, which depended on its mass-to-charge (m/z) ratio. Finally, the pure peptide was obtained and subjected to lyophilisation and biological activity assay.

Besides, some useful bioinformatics websites can be utilised in prediction and analysis of the physicochemical properties and secondary structure of the peptide,

such as HELI-QUEST (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py) and NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6 Antimicrobial assays

Three standard microorganisms were used in these assays including the Gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*, NCTC 10788), the Gram-negative bacterium, *Escherichia coli* (*E.coli*, NCTC 10418) and the pathogenic yeast, *Candida albicans* (*C. albicans*, NCPF 1467), to assess antimicrobial and bactericidal activity of the peptide. The MIC assay is designed to test the minimum visible growth-inhibitory concentration of microorganisms and is reliable, rapid and inexpensive. The MBC assay is designed to test the minimum concentration of antibiotic that is lethal to a microorganism *in vitro.*

2.6.1 Microorganism inoculation

1) Beads of each of the three microorganisms were removed from the -20℃ freezer and placed into McCartney bottles of 100 ml Muellar Hinton Broth.
2) The flasks with beads were incubated in an orbital shaking incubator (Stuart, UK) overnight (16 - 20h) at a speed of 150 rpm at 37℃. For each microorganism, two McCartney bottles with 20ml MHB were placed into a 37℃ incubator to be

warmed up overnight.

2.6.2 Peptide preparation

The sample was initially dissolved in a stock solution of 1024 μM and subsequently double-diluted to achieve final concentrations of the peptide from 512 to 1 μM.

2.6.3 Subculture

1) The next day, 500 μl of each microorganism culture were transferred into pre-warmed McCartney bottles with MHB and replaced into the 37℃ shaking incubator until the logarithmic phase of microorganism growth was reached. (Table 2.6.3.1).

Table 2.6.3.1 The appropriate OD values for the three microorganisms used.

Organism	Subculture incubation time	OD	Concentration (cfu/ml)
S. aureus	1.5 h	0.2	10^8
E. coli	1.0 h	0.4	10^8
C. albicans	2.0 h	0.15	10^6

*The OD was measured by a UV spectrophotometer at λ=550nm.

* cfu= colony-forming units.

2) 100 μl of *S.aureus* and *E.coli* subcultures were added into 19.9 ml fresh

pre-warmed MHB and mixed well by pipetting. The *C.albicans* subculture was ready to use. All three microorganism subcultures were used at 10^6 cfu /ml.

2.6.4 Minimum inhibitory concentration (MIC) measurements

1) Samples and controls were arranged in the 96-well plate with 5 replicates of each.

   Control: 100 µl sterile MHB

   Samples: 50 µl peptide solution + 50 µl culture

   Blank: 100 µl culture

2) The 96-well plate was placed in the shaking incubator for 5 min to make the mixture well-distributed and was then incubated at 37℃ overnight.

3) The next day, the inhibition of microorganism growth was measured by the absorbance at 550 nm using the Synergy HT plate reader (BioTek, USA).

2.6.5 Minimum bactericidal concentration (MBC) measurements

1) 10 µl of supernatant from each clear well on MIC plates, were dropped onto the MHA plate. The MHA plates were then incubated at 37℃ overnight.

2) The next day, the plates were examined for microorganism growth. The MBC value was obtained as that in which no colonies grew at the lower concentration.

2.7 Haemolysis assay

2.7.1 Peptide and control preparation

1) Synthetic peptide was weighed and dissolved in sterile PBS to a concentration of 1024 μM
2) This was then double-diluted to achieve the following concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 μM. The peptide solutions were kept in centrifuge tubes until required.
3) 22 μl Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) were diluted in 1078 μl PBS solution as a positive control in which 1% (v/v) non-ionic detergent Triton X-100 (Sigma-Aldrich) can produce a 70% haemolytic effect.
4) PBS solution was regarded as a negative control (blank control) for the comparison of non-haemolytic effects.
5) 22 μl DMSO was diluted in 1078 μl PBS solution as a vehicle control to assess the influence of 1% (v/v) DMSO solution on the erythrocytes.

2.7.2 Horse blood preparation

Two ml of fresh defibrinated horse blood (TCS Biosciences Ltd, Buckingham, UK) were transferred into a 50 ml universal tube and centrifuged at 100 × g for 5 min to separate the serum and erythrocytes. Then, the cloudy supernatants were discarded completely and 30 ml of PBS solution were added. Several washing steps were repeated until the supernatants were clear. Then, the supernatants were discarded as

cleanly as possible and PBS solution was refilled to the 50 ml volume. Finally, gentle shaking was needed to obtain an even 4% (v/v) erythrocyte suspension.

2.7.3 Haemolysis assay

1) 200 µl of peptide sample solution were added into a sterile tube with 200 µl of red blood cell suspension. 5 replicates were made for each peptide concentration.
2) 200 µl of Triton solution were added into a sterile tube with 200 µl of red blood cell suspension as a positive control (100% haemolysis). 5 replicates were made for this.
3) 200 µl sterile PBS was added into a sterile tube with 200 µl of red blood cell suspension as negative control (blank 0% haemolysis). 5 replicate were made for this.
4) The tubes were incubated for 120 min at 37℃.
5) After incubation, all tubes were centrifuged at 100 × g for 5 min. 200 µl of supernatant from each tube were transferred to a 96-well plate.
6) The absorbance at 550 nm was measured using a Synergy HT plate reader (BioTek, USA).
7) The percentage of haemolysis was calculated using the following formula and a graph was drawn. The haemolysis assay was repeated at least three times and the SEMs of three experiments were calculated to show the variability and repeatability.

$$\text{Haemolysis\%} = (A-AO) / (AX-AO) * 100\%$$

Where A represents the OD (λ550) of peptide/ erythrocyte mixture, AX the OD (λ550) of the positive control and AO the OD (λ550) of the negative control.

Chapter 3

Results

Results

3.1 "Shotgun" cloning of novel peptide biosynthetic precursor-encoding cDNA

Using the molecular cloning strategy as described in section 2.1, a cDNA encoding the biosynthetic precursor of a new peptide was consistently cloned from the skin secretion library. The nucleotide and translated open-reading frame amino acid sequence of this cDNA are shown in Figure 3.1.

```
     M  A  F  L   K  K  S   L  F  L   V  L  F  L   G  L  V
   1 ATGGCTTTCT TGAAGAAATC TCTTTTCCTT GTACTATTCC TTGGATTGGT
     TACCGAAAGA ACTTCTTTAG AGAAAAGGAA CATGATAAGG AACCTAACCA
      S  L  S   I  C  E  E   E  K  R   E  T  D   E  E  E  N
  51 TTCTCTTTCC ATCTGTGAAG AAGAGAAAAG AGAGACAGAT GAAGAAGAAA
     AAGAGAAAGG TAGACACTTC TTCTCTTTTC TCTCTGTCTA CTTCTTCTTT
        D  Q  E   E  D  E   Q  S  E  E   K  R  F   L  S  L
 101 ATGATCAAGA GGAAGATGAG CAAAGTGAAG AGAAGAGATT CTTGAGCTTG
     TACTAGTTCT CCTTCTACTC GTTTCACTTC TCTTCTCTAA GAACTCGAAC
      I  P  K  I   A  T  G   I  A  A   L  A  K  H   L  G  *
 151 ATACCAAAAA TAGCAACTGG AATAGCTGCA CTTGCTAAAC ATTTAGGTTA
     TATGGTTTTT ATCGTTGACC TTATCGACGT GAACGATTTG TAAATCCAAT

 201 ATACAATGTA ACATTTCATA ACTCTAAGGA GCACAATTAT CAATAATTGT
     TATGTTACAT TGTAAAGTAT TGAGATTCCT CGTGTTAATA GTTATTAACA
 251 TCTCAAAATA CATTAAAGCA TATTTAACCA ACAAAAAAAA AAAAAAAAAA
     AGAGTTTTAT GTAATTTCGT ATAAATTGGT TGTTTTTTTT TTTTTTTTTT
 301 AAAAAAA
     TTTTTTT
```

Figure 3.1 Nucleotide and translated open-reading frame amino acid sequence of biosynthetic precursor cDNA encoding the novel mature peptide. The putative signal peptide is single-underlined, the mature peptide is double-underlined, and the stop codon is indicated by an asterisk.

3.2 MALDI-TOF-MS of QUB1977

The synthetic QUB1977 was analysed by MALDI-TOF mass spectrometry. The major peptide ion observed at m/z 1977.11, was consistent with the desired sequence（Figure 3.2）.

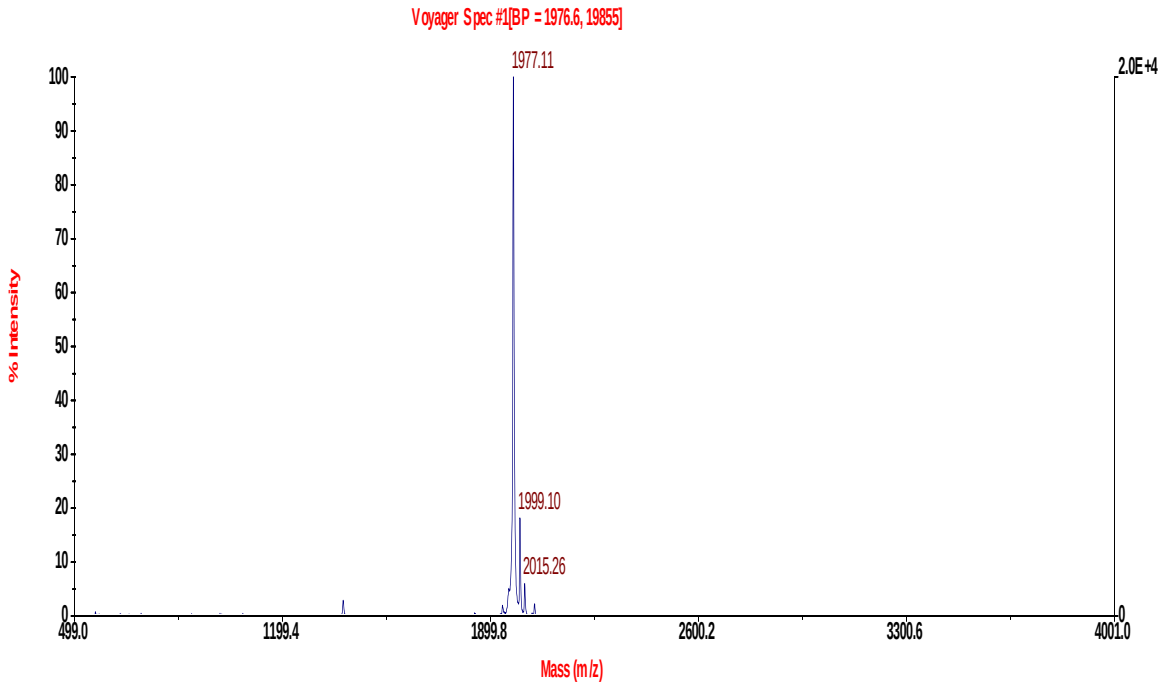

Figure 3.2 MALDI-TOF mass spectrum of synthetic QUB1977

3.3 Secondary structure prediction by HELI-QUEST

The physiochemical properties and structure of QUB1977 were preliminarily predicted by the online tool HELI-QUEST. This peptide contained two positively charged residues (Lysines), a hydrophobic face and twelve nonpolar residues constituting almost two thirds of the residues.

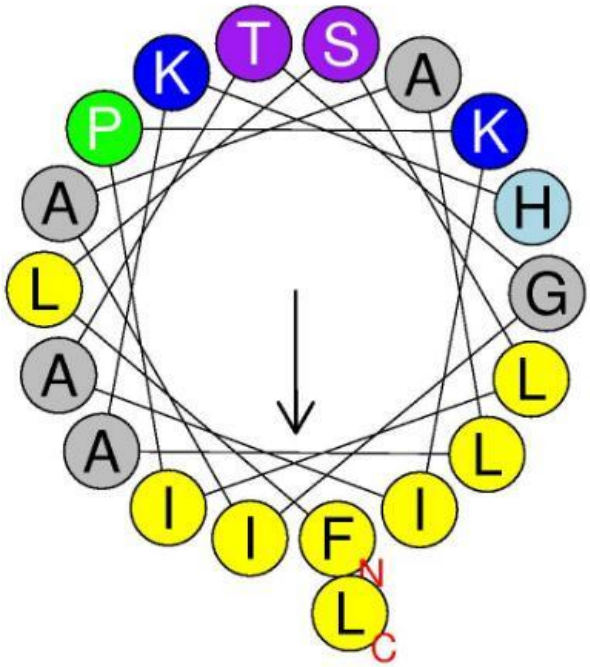

Figure 3.3 Predicted secondary structure of QUB1977 using HELI-QUEST

3.4 Antimicrobial assays

The synthetic replicate of QUB1977 exhibited effective growth inhibition of the Gram-positive bacrerium, *S.aureus* (Figure 3.3.1), the Gram-negative bacterium, *E.coli* (Figure 3.3.2) and the yeast, *C.albicans* (Figure 3.3.3). MICs were 4, 32 and 4 μM, respectively. Their corresponding MBCs were 8, 128 and 4 μM, respectively.

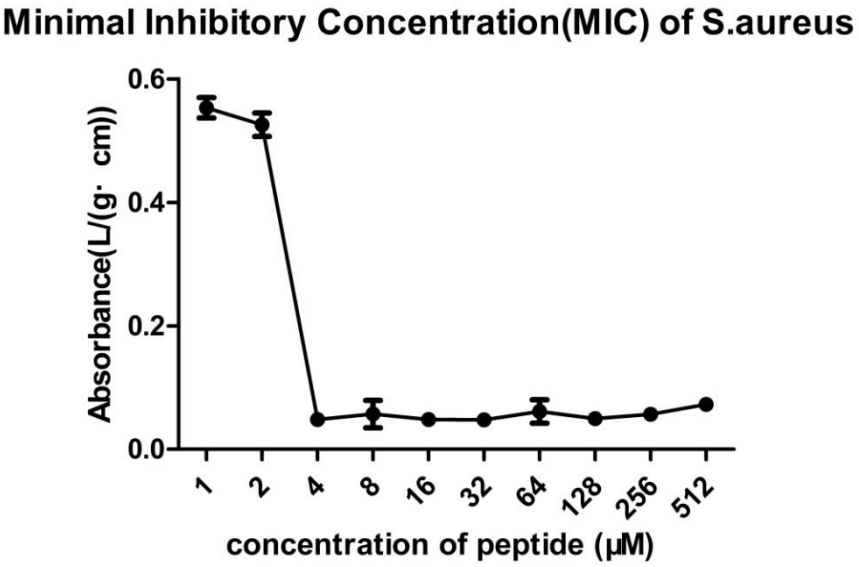

Figure 3.4.1 MIC of QUB1977 against *S. aureus* (λ =550 nm)

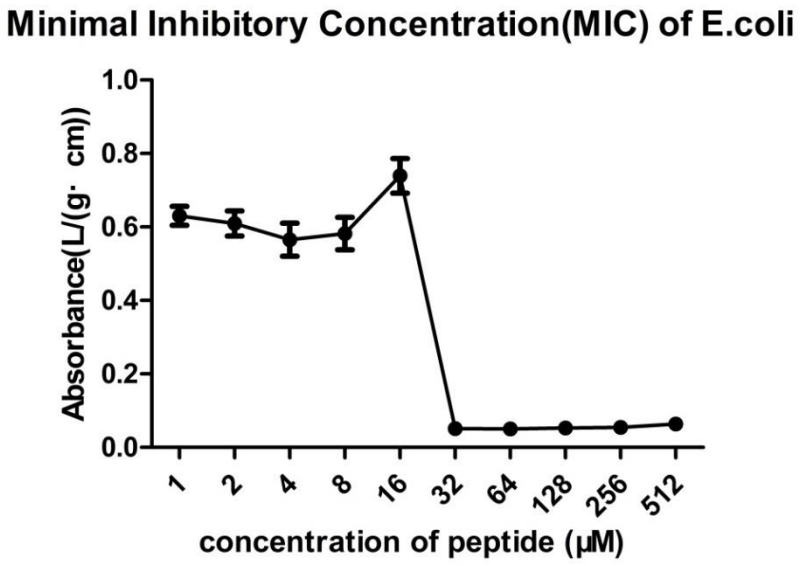

Figure 3.4.2 MIC of QUB1977 against *E. coli* (λ=550 nm)

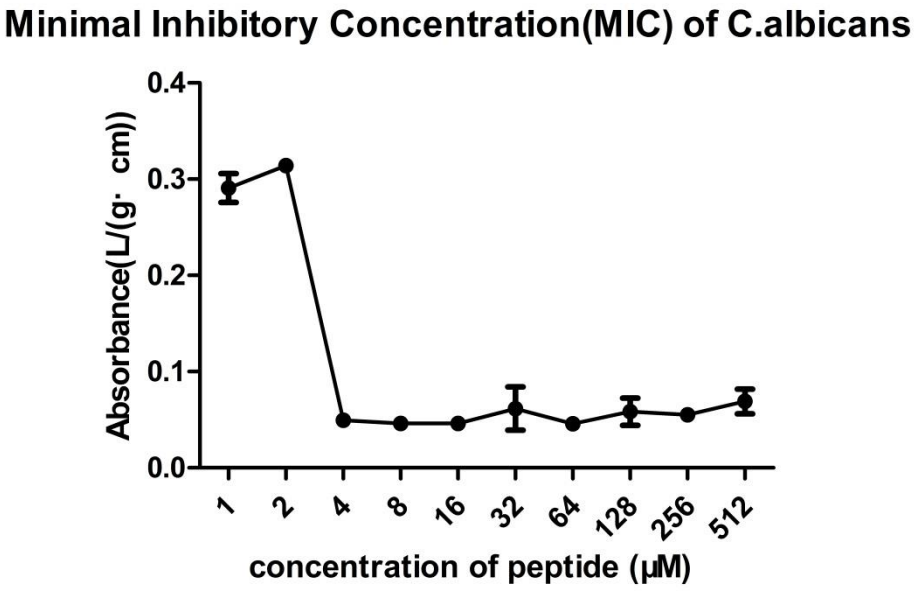

Figure 3.4.3 MIC of QUB1977 against *C. albicans* (λ=550 nm)

3.5 Haemolysis activity

The haemolysis assay showed that the concentration of the test peptide which induced 50% haemolysis (HC_{50} value) was 23 μM (Figure 3.4). The assay was repeated 3 times, each of 5 replicates.

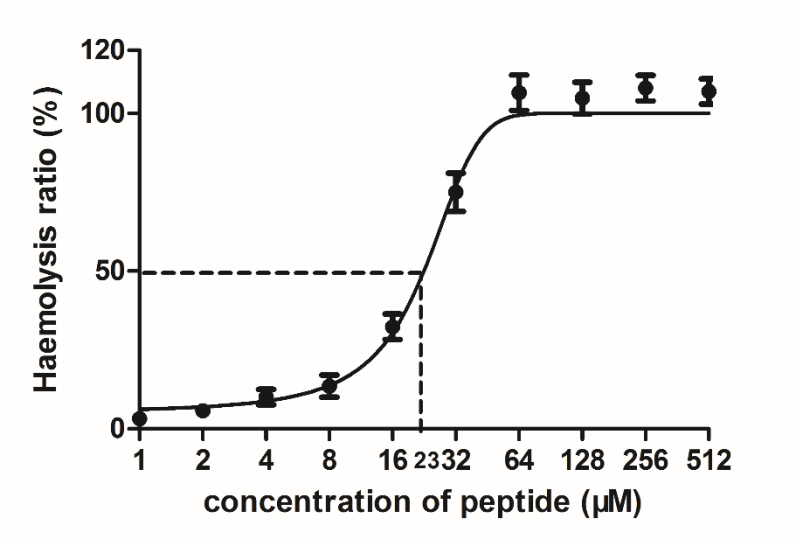

Figure 3.5 Haemolytic activity of QUB1977

Chapter 4

Discussion and Conclusion

Discussion and conclusion

Amphibian skin has been considered as a “treasure store” of bioactive peptides on which numerous research groups have focused their attention. Some advanced technologies and methodologies like high throughput molecular techniques involving *de novo* peptide sequencing via tandem mass spectrometry, cDNA cloning, pharmacological screening, and surface plasmon resonance applied to peptide discovery, have led to fast structural data acquisition and the generation of peptide molecular libraries (de Azevedo Calderon, 2011). Based on the current plight of many of the amphibians worldwide, the method of extracting and identifying the novel peptide from the skin secretion used in this work is highly favorable. This high throughout method can identify the skin secretion components without sacrificing the specimens.

Generally, the therapeutic potential of the host-defence peptides from frog skin secretions has not been fully realised since many peptides have multifunctional activities such as anti-cancer, anti-viral, immunomodulatory and as anti-diabetic agents (Conlon et al., 2014). Among all these peptides, antimicrobial peptides (AMPs) have attracted the most attention for their potential as agents against multidrug-resistant pathogens. AMPs that have been escalating as biological weapons in living organisms over millions of years, with no doubt, constitute part of the immune system of multicellular creatures. Since the dose of killing or inhibiting bacteria is not very large—normally at micromolar concentrations and AMPs do not

interact with specific receptors, it is rare for these to induce drug resistance.

However, unless a long-acting, non-toxic analogue can be developed, the rapid clearance of the peptide in the circulatory system will keep preventing the possibility of systemic administration. Peptides used in the form of sprays or ointments for infection or damage of the skin can penetrate into the stratum corneum to kill the microorganisms. In this case, future therapeutic applications would tend to be administered topically like the treatment of diabetic foot ulcers, impetigo, and to promote wound healing.

Sequence of the QUB1977 was compared with other known peptides by NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of QUB1977 was similar with Phylloseptin-2 and Phylloseptin-PT from *Phyllomedusa tarsius* with 95% identities. Therefore, QUB1977 was considered as a member of phylloseptin family.

Many studies have proven that phylloseptins show antimicrobial activity against Gram-negative bacteria: *Acinetobacter calcoaceticus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Leite et al., 2005; Resende et al., 2008); Gram-positive bacteria: *Enterococcus faecalis*, *Klebsiella peneumoniae*, *Staphylococcus aureus*, and *Streptococcus agalactiae*; Fungi: *Candida albicans* (Resende et al., 2008); and Protozoa: *Leishmania amazonensis* (promastigotes) (Ku ̈ckelhaus et al., 2009),

Plasmodium falciparum (rings, trophozoites, and schizonts) (Ku ̈ckelhaus et al., 2009), and *Trypanosoma cruzi* (trypomastigotes) (Leite et al., 2005).

As can be seen here, this novel AMP of the phylloseptin family, from the skin secretion of *Phyllomedusa camba*, displayed broad-spectrum activity against the Gram-positive bacterium, *Staphylococcus aureus*, the Gram-negative bacterium, *Escherichia coli* and the yeast, *Candida albicans*, with MICs of 4, 32 and 4 μM, respectively.

This observation was consistent with the finding from other phylloseptins that peptides from this family show a biased selectivity for prokaryotic over eukaryotic membrane targets. This is because prokaryotic cell membranes contain relatively more negatively charged phospholipids at the outer leaflet of the membrane bilayer (Zasloff, 2002). Thus, the appropriate application of the peptide studied here would be to reduce the numbers of Gram-positive and Gram-negative bacteria.

The classical mechanism of AMPs involves cell membrane damage (Epand & Vogel, 1999). The cationic compounds can interact with the negatively charged cell membrane or some AMPs destroy the cell membrane integrity by specifically interacting with the membrane compounds (Hancock, 2001). In addition to the physicochemical features of the lipids on the membranes of microbial targets, the composition and secondary structures of AMPs are also responsible for their effects.

This novel peptide, QUB1977, was analysed by an on-line tool named HELI-QUEST to obtain a preliminary idea of its secondary structure and some physicochemical characteristics. The peptide of 19 amino acid residues was typically cationic and contained an amphiphilic α–helix. The amphiphilic nature of this peptide was presumed to explain its biological activity through electrostatic interactions of the positively charged ions association with negatively charged cytoplasmic membranes subsequently leading to the disrupted cell membrane.

The peptide QUB1977, shares the same structural characteristics with other phylloseptins, namely, a highly conserved sequence FLSLI[L]P in the N-terminal region and C-terminal amidation which has been shown to promote biological activity (Leite et al., 2005; Ali et al., 2001; Katayama et al., 2002). Nevertheless, the biological activity of phylloseptins may change radically if the sequence of model compounds has been slightly modified even though the total amino acid composition is constant (Wieprecht et al., 1997). This is because peptides which have slight modifications with an increased hydrophobic moment are considerably more active in interactions between lipid acyl chains and the hydrophobic helix core during the membrane-permeabilising process even though the number and positions of charged residues are constant.

What is more, members of the phylloseptin family normally exhibit a weak effect on

red blood cells (Leite et al., 2005) and display some toxic effects on mammalian cells only at very high concentrations (Ku¨ckelhaus et al., 2009). For instance, phylloseptin-1 (PSN-1), from *Phyllomedusa sauvagei* skin secretion, had no haemolytic activity at the tested concentrations against *S.aureus* (Zhang, 2010).

While in this assay, a significant haemolytic activity (EC_{50} of 23 μM) was demonstrated, it is concluded that the peptide here may become a lead compound for future drugs against Gram-positive bacteria and yeasts instead of against Gram-negative bacteria （MIC of *E.coli*: 32 μM）.

Haemolysis is a common feature of many AMPs and is an unfavorable effect that hinders their use in systemic therapy. However, this problem can be solved by rationally designing structural analogues of major molecules, which is common in drug development.

On the other hand, due to the ever-changing revisions of species taxonomy in anurans, some confusion may be caused. For example, some publications named the peptides according to their evolutionary origin or functional similarities while some chose to name the peptides by initial letters from the name of the species, all of these making the situation more complicated. Therefore, it is highly recommended that there should be a consistent nomenclatural system not only for AMPs but also for peptides which are active as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents

(Conlon et al., 2014).

Some existing nomenclatural systems can be referred to, such as the online reference from Frost (Frost, 2014) or a practical nomenclatural system proposed by Conlon (Conlon, 2008). Besides, the Basic Local Alignment Search Tool (BLAST) can help to compare similarity with existing peptides.

Ultimately, AMPs are highly likely to be useful therapeutic tools because they are not only able to kill bacteria, but also viruses and fungi. In addition, their antimicrobial activity is exerted due to their multifunctional properties. This feature makes microbial resistance more difficult to develop. Today, our in-depth research will further develop the knowledge in this field and highlight the value of AMPs as potential therapeutic agents.

Future work

AMPs are promising as alternatives to antibiotics in the near future because of their properties of killing pathogenic microorganisms and they will absolutely have a positive effect on human life expectancy. It will soon become a reality that people manage the design and use of AMPs against various antibiotic-resistant bacterial pathogens.

There are some methods that may help this peptide to become more effective in antimicrobial therapy and show a relatively low level of haemolytic activity at the

MIC range:

AMPs can work with traditional antibiotics.

A variety of AMPs can work together as a cocktail agent.

Make structural modification on AMPs.

In conclusion, a thorough understanding of the correlation between the structure and function of AMPs will help rationally design cheaper and more effective analogues (Hwang & Vogel, 1998; Hyberts, 1992). Through some specific post-translational modifications including amidation, disulphide-bonding, L-to-D amino acid isomerisation, tyrosine-sulphation, proline-hydroxylation and amino terminal pyroglutamate formation and so on, new functions of AMPs may develop and cytotoxicity may be reduced. Therefore, further work needs to be done on the relationships between the structures and the functions of this class of peptide molecule.

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